

Behavioral effects of long-chained ketones in *Vipera ammodytes*: an experimental approach

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Abstract

Snakes heavily depend on chemical communication and use chemical cues for trailing, foraging, searching for mates, and other behaviors. Snake pheromones reported so far are complex mixtures, consisting mainly of heavy, long-chained ketones. However, such ketones cannot be detected in the sex pheromones of some species, and for others, airborne compounds are suggested to participate in intersexual communication. Thus, to establish whether a certain compound is part of the snake sex pheromone, behavioral assays should be performed. The most frequently found ketones in the skin extracts of *Vipera ammodytes* (Linnaeus, 1758) are 2-pentacosanone and 2-heptacosanone, which are hypothesized to participate in intraspecific communication. In the present study, we test male and female individuals' responses to paper towels soaked with these two ketones (separately and together), *n*-hexane, heptacosane, and a control. We suggest that the ketones produced by *V. ammodytes* have a role in intraspecific communication and demonstrate that they elicit specific courtship-related behaviors in males, but not in females. Our results further show that 2-pentacosanone and 2-heptacosanone evoke sexual attraction, with their combination proving more effective than either compound alone, although the modest response suggests that other compounds may also be involved. We suggest that there are additional compounds, of a different nature, that most likely constitute the female sex pheromone. We hypothesize that males can also produce these ketones, which may provide them with a mating advantage by emitting false cues to rivals.

Key Words

communication, mating, pheromones, sex, snakes

Introduction

Due to the lack of limbs, snakes are, to some extent, constrained in the visual perception of their environment (Walls 1942). Although some species, like the Eastern

Fox Snake, *Pantherophis vulpinus* (Baird & Girard, 1853), prefer visual to chemical perception for foraging and other activities (Saviola et al. 2012), most snakes rely strongly on chemical cues for communication (Mason et al. 1989; Ford and Burghardt 1993; Mason and Parker

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2010). Snakes use chemical cues for trailing conspecifics, locating winter hibernacula, and finding opposite-sex individuals during the mating season (Ford 1986; Mason et al. 1989, 1990; Greene et al. 2001; LeMaster et al. 2001). Snake semiochemicals are part of their integumental skin lipids (Garstka and Crews 1981; Mason et al. 1989), and their pheromones are generally considered to contain a mixture of heavy, non-volatile, long-chained ketones (Mason et al. 1987, 1989). However, in recent years, several studies have suggested that airborne compounds play a significant role in sexual chemical communication (Aldridge et al. 2005; Shine and Mason 2011; Jellen et al. 2022). Mason et al. (1989) describe the sex pheromones of the red-sided garter snake, *Thamnophis sirtalis parietalis* (Linnaeus, 1758), as a mixture of 17 long-chained saturated and unsaturated methyl ketones (C29–C37), with females thought to attract males for courtship using their specific pheromones. It appears that unsaturated ketones are more attractive to males than saturated ones (Mason et al. 1990). Interestingly, some males of the species produce pheromones similar to those of females, which may provide them with a mating advantage by presenting false cues to rivals (Shine et al. 2000). Notably, larger females have higher amounts of unsaturated ketones in their skin secretions, making them more attractive (LeMaster and Mason 2002). However, no methyl ketones were found in female samples of the brown tree snake, *Boiga irregularis* (Bechstein, 1802), and its pheromone mixture is thought to consist of other compounds (Greene and Mason 1998). Although there are studies on other species such as the indigo snake, *Drymarchon corais* (Boie, 1827) (Ahern and Dowling 1974), *Boiga irregularis* (Murata et al. 1991; Greene and Mason 1998), and species of the genus *Crotalus* (Schell and Weldon 1985; Weldon et al. 1992), and the common watersnake, *Nerodia sipedon* (Linnaeus, 1758) (Jellen et al. 2022), most chemical studies on pheromone activity in snakes focus on the genus *Thamnophis* (e.g., Mason et al. 1989, 1990; Shine and Mason 2011). Therefore, the field of pheromone communication in snakes remains understudied.

In addition, studies on chemical communication in snakes are geographically biased, with almost no Old World species investigated and most research focused on New World species (e.g., Ahern and Dowling 1974; Mason et al. 1989, 1990; Murata et al. 1991; Shine and Mason 2011). There are, however, studies on intraspecific communication or trailing behavior of vipers in Europe (Andrén 1986; Andrén and Nilsson 1983; Shine 1978), although these do not address the identification of sex pheromone components.

European vipers exhibit highly specific mating rituals in which males are much more active than females (Andrén 1986; Andrén and Nilsson 1983). Prior to courtship, males usually engage in “ritualized” male-to-male combats for access to a female. Initially, both males raise their heads high above the ground. If neither retreats, the combat begins with straight and spiral body movements in an elevated posture, with both snakes pressing their bodies tightly together while attempting to dominate the opponent

by pushing his head down onto the ground (Andrén 1986). If this fails, the combat continues with horizontal entwining and spiral twisting, accompanied by extensive body contact while the snakes slowly move forward (Andrén 1986). This stage includes violent, back-and-forth body movements, concluding with a sudden, forceful thrust. The combat ends when the weaker male is forced to retreat. The victor then courts the female using a series of tongue flicks, head nods, and chin rubs along the female’s body; the courtship also includes tail vibrations from both sexes (Andrén 1986; Andrén and Nilsson 1983; Shine 1978).

The skin lipids of *Vipera ammodytes* (Linnaeus, 1758) have recently been investigated (Andonov et al. 2020, 2023), but the function of the identified compounds remains unknown. To determine their function, behavioral assays are necessary.

Skin lipid extracts of *V. ammodytes* contain long-chained, non-volatile ketones (Andonov et al. 2020, 2023), similar to those found in the pheromone complexes of other species (e.g., Mason et al. 1989). Among the various ketones found in this species, 2-pentacosanone and 2-heptacosanone are the most frequently occurring heavy, long-chained methyl ketones and are likely involved in chemical communication, while other ketones are relatively rare (Andonov et al. 2020, 2023). However, because methyl ketones are not always present in snake pheromones (e.g., Greene and Mason 1998), this hypothesis requires confirmation through behavioral experiments. Additionally, it should be noted that both males and females produce these ketones in similar concentrations (Andonov et al. 2020, 2023).

In the present study, we test, through behavioral experiments, whether the most common ketones detected in *V. ammodytes* (Andonov et al. 2020, 2023) act as pheromones and are involved in intraspecific communication. Behavioral experiments on chemical communication in snakes have been conducted over the years for various species. Depending on their goals and hypotheses, these studies use decision-based trail-following tests with Y-shaped or similar mazes (e.g., Ford and Low 1983; Greene et al. 2001; LeMaster and Mason 2001), tongue-flick rates, or observations of courtship behavior in terraria (Mason et al. 1990; Greene and Mason 1998). During initial trials, we constructed Y-shaped labyrinths for trailing experiments. However, they did not yield useful results, as males displayed mainly exploratory behavior regardless of the presented compound, and females rarely moved (Andonov et al., unpublished data). Additionally, we observed that vipers easily became stressed when moved from their terrarium to an unfamiliar space, such as the Y-maze, which significantly affected their behavior. To reduce human interference, we avoided the commonly used cotton swab test (Burghardt 1967, 1969; Cooper 1994; Cooper et al. 2000; Van Moorlegheem et al. 2020) and direct observation. Instead, we used video recordings and exposed individuals to paper towels treated with various compounds (Mason et al. 1990), allowing for more natural behavioral responses.

Through a series of behavioral experiments, we investigate which compounds elicit courtship-related behaviors and attempt to determine whether both sexes are attracted to these compounds or if sexually dimorphic responses are present. With this study, we aim to explain the lack of observed differences in ketone production between male and female *V. ammodytes*.

Materials and methods

Test animals

We collected a total of eight male and seven female adult individuals of *Vipera ammodytes* in April 2022 (three males and three females) and April 2023 (five males and four females) from a single population near the village of Karlukovo, NW Bulgaria (43°10'N, 24°3'E [DDM]). The animals were tested in the same year of collection and kept in captivity for no longer than two months. We obtained standard morphometric measurements—snout–vent length (SVL) and tail length (TL)—for each individual using a measuring tape (precision: 0.5 cm). Snakes were weighed using a digital scale (precision: 0.01 g). Sex was determined by gentle palpation to check for the presence or absence of hemipenes and by examining tail length, tail root width, and number of subcaudal scales (Tomović et al. 2002). Individuals with total body length (SVL + TL) > 46 cm in females and > 49 cm in males were considered adults (Dyugmedzhiev et al. 2020).

The vipers were kept in captivity for two months in a specially equipped laboratory at the Institute of Biodiversity and Ecosystem Research. Each animal was housed individually in a terrarium (50 × 30 × 30 cm) with beech chips as substrate, a continuously running heating coil at one end, and a shelter at the other. The temperature was maintained between 30 °C and 32 °C during the day (09:00 h to 20:00 h) and approximately 17 °C at night. Each viper was fed every 10–15 days with a dead laboratory mouse. Water was provided *ad libitum*.

The research was conducted in accordance with permit No. 861/13.01.2021 issued by the Ministry of Environment and Waters. No animals were harmed or showed signs of deteriorating health; none were euthanized or died during the study period. At the conclusion of the experiments in both years, all vipers were released at their exact site of capture.

Behavioral assays

Prior to the start of the tests, all individuals were kept for one week in the terrariums so they could adapt to the new environment before starting the experiments. Thereafter, we performed a series of behavioral assays with each individual. All tests were conducted from the beginning of May to the first ten days of June, which falls within the mating period of *V. ammodytes* (Beshkov 1977; Stojanov

et al. 2011; Dyugmedzhiev 2020). We tested each viper by placing a paper towel soaked with a specific compound in its terrarium in front of the tested individual, following the procedure described in Mason et al. (1990). Thus, the total number of behavioral assays was 270 tests (144 tests with males and 126 with females). The paper towel was kept for 10 min, and during this period, reactions of the viper were recorded with a video camera (Sony HANDY-CAM HDR-PJ410, 9.2 megapixels) positioned in front of the terrarium, without human presence in the room. Different synthetic compounds were presented to the individuals, as well as control tests with no compounds on the paper towel. Between 0.20 and 0.25 ml of a single test compound was dripped onto the towel with a pipette (i.e., each solution was tested separately). We repeated each of the behavioral tests three times (for every individual with each different compound) for statistical purposes and a good representative sample size. We performed no more than four tests per day per animal, with at least an hour between the tests, and there was a minimum of one day between the days each animal was tested.

For the tests, we used synthetic *n*-hexane (C₆H₁₄) – “hexane,” heptacosane (C₂₇H₅₆) – “C27 alkane,” 2-pentacosanone (C₂₅H₅₀O) – “C25 ketone,” 2-heptacosanone (C₂₇H₅₄O) – “C27 ketone,” and a mixture of equal parts 2-pentacosanone and 2-heptacosanone – “C25–C27 ketones.” All synthetic compounds were chosen based on available literature data for the species (see Andonov et al. 2020, 2023) and bought from laboratory suppliers in solid aggregate state (except the liquid *n*-hexane). In the laboratory, we dissolved the solid compounds in *n*-hexane to a 1 mg/ml concentration (Mason et al. 1990), and the *n*-hexane used for the behavioral tests was 99% purity.

The compounds were presented to each individual in a random order. The substrate was not contaminated by the tested compounds, and the terrarium was ventilated for a few minutes after each procedure. After performing the tests, we watched every video recorded and transcribed full ethograms for the behavior of the individual during the experiment. We counted the tongue-flicks for the whole duration of the experiment (10 min) and calculated the general tongue-flick rates (GTF)—the total amount of tongue-flicks divided by the duration of the experiment—and specific tongue-flick rates (STF)—the total amount of tongue-flicks divided by the time elapsed between the first tongue-flick and the last. We also recorded the time that each individual spent lying still before the first tongue-flick. If the individual moved out of the filming frame, the “out-of-sight” duration was subtracted, respectively, from the total of 10 min or the period between the first and the last tongue-flicks. We recorded the presence or absence of interest in the compound dripped on the paper towel (INT). A positive response was registered if the individual displayed interactions with the paper towel and focused their tongue-flicks on it for at least 10 seconds. We observed chin-rubbing behavior (RUB), which is typical of mating male individuals (Andrén 1986; Andrén and Nilson 1983; Shine 1978). A positive response

was recorded if the rubbing continued for more than 10 seconds consecutively (Mason et al. 1990), and we counted the number of these responses.

Although there are functional differences in the types of tongue-flicks in terms of the tongue's extension and the flicks' frequency (Gove and Burghardt 1983; Daghfous et al. 2012), the type of experiment did not allow us to differentiate between the different types of tongue-flicks. In addition, the followed methodology does not make a difference between the types of tongue-flick, and therefore, we counted each tongue-flick for the respective variables.

Statistical analyses

For the statistical analyses, we used R (R Core Team 2024) and its packages “brms” (Bürkner 2021), “emmeans” (Lenth 2025), “loo” (Yao 2017), and “mvabund” (Wang et al. 2012). Data were tested for normality using a Shapiro–Wilk test, and the null hypothesis was rejected. Descriptive statistics were performed for each variable. Considering the different types of data of the different variables (INT – binary, RUB – discrete, and GTF and STF – continuous), the distribution of each variable was tested separately. We first calculated the proportion of zero values for each variable, the excessive amount of which (see Results) directed us to test zero-inflated models. We then fitted hurdle models for GTF and STF using both gamma and lognormal distributions and compared them using the Watanabe–Akaike information criterion (WAIC) and, if necessary, with leave-one-out cross-validation (LOO), which indicated that the hurdle gamma model was preferred for both GTF and STF. For INT, we compared standard binomial and zero-inflated binomial models, choosing the latter. Lastly, for RUB, we compared Poisson and zero-inflated Poisson models, with the zero-inflated Poisson model being preferred based on WAIC.

We used a Bayesian multivariate generalized (non-)linear mixed-effects model (GLMM), using the multivariate model formula (mvbrmsformula) in the “brms” package in R, where sex and compound were set as predictors and individual as a random effect. The tests were set with four chains (independent sequences of samples), 4000 iterations per chain (steps taken by the Markov Chain Monte Carlo algorithm), and an adapt_delta of 0.95 (a tuning parameter controlling the target acceptance rate for the sampler). This was followed by a pairwise comparison by estimated marginal means, with the function “emmeans” in R (Lenth 2025). The whole sample was tested first, and thereafter, based on the results, male assays and females were tested separately with multivariate GLMM, followed by a pairwise comparison. In addition, the compounds were grouped as follows: 1) ketones—C25 ketone, C27 ketone, and C25–C27 ketones; 2) other—hexane, C27 alkane; 3) control—the control group, and further tests for males and females among the compound groups were performed (multivariate GLMM and pairwise comparison).

Results

The average time for an individual to react and make the first tongue-flick was 291 seconds ($n = 128$, min–max = 1–593; $SD = 162.2$) after the start of the experiment. Only tests with individuals who exhibited tongue-flicking behavior were included in this statistic.

We first calculated the proportion of zero values for each variable. For interest in the compound (INT), chin-rubbing (RUB), general tongue-flick rates (GTF), and specific tongue-flick rates (STF), the proportions were, respectively, 81.85%, 92.96%, 52.59%, and 52.59%. Males showed higher levels of INT in response to the two ketones, both separately and together, while females expressed such behavior very rarely and only for the C27 ketone (Fig. 1). Chin-rubbing behavior was observed only in males, and the most active individuals (those performing more than two chin-rubbings) again reacted to the ketones (Fig. 2). Note that in terms of the number of individuals presenting this behavior, the different compounds did not produce very diverse results. The GTF was significantly higher in males, with the strongest reactions observed toward C25 ketone and C27 ketone, although these were not significantly different from the other compounds (Fig. 3). The situation was similar for STF, where the highest result for males was recorded for C25 ketone (Fig. 4). Complete descriptive statistics for each variable are provided in the supplement (Suppl. material 1).

For INT, both WAIC and LOO indicated negligible differences between the standard binomial and the zero-inflated binomial models (WAIC difference = 0.3; LOO difference = 0.3), so the binomial distribution was used. For RUB, the zero-inflated Poisson model performed notably better than the Poisson model (WAIC difference = 11.6; LOO difference = 11.3). The results for GTF indicated a better fit for the hurdle gamma model (WAIC difference = 7.0) compared to the gamma model. Posterior predictive checks were performed for both models, confirming their overall fit. The hurdle gamma model also showed a notably better fit for STF compared to the gamma model (WAIC difference = 12.1; LOO difference = 12.1) and was therefore used in subsequent analyses. Posterior predictive checks confirmed the fit of both models.

The multivariate GLMM showed that males and females differed in terms of INT and RUB, with positive estimates and confidence intervals (CI) for males: INT (estimate = 96.13; lower 95% CI = 5.40; upper 95% CI = 324.83) and RUB (estimate = 1231.17; lower 95% CI = 93.47; upper 95% CI = 3456.11), indicating that males exhibited more active behavior toward the compounds. The model also showed that the individual (random effect) had a statistically significant influence on the results, with positive CIs for INT (intercept: $SD = 1.03$; 95% CI = 0.41–2.00), RUB (intercept: $SD = 3.17$; 95% CI = 1.41–6.48), GTF (intercept: $SD = 0.81$; 95% CI = 0.41–1.29), and STF (intercept: $SD = 0.36$; 95% CI = 0.07–0.81). However, the model showed convergence issues, with divergent transitions and R -hat values > 1.1 . Therefore, we did not interpret the results further and instead continued with separate analyses for males and females. The influence of individual variation was confirmed in the separated models.

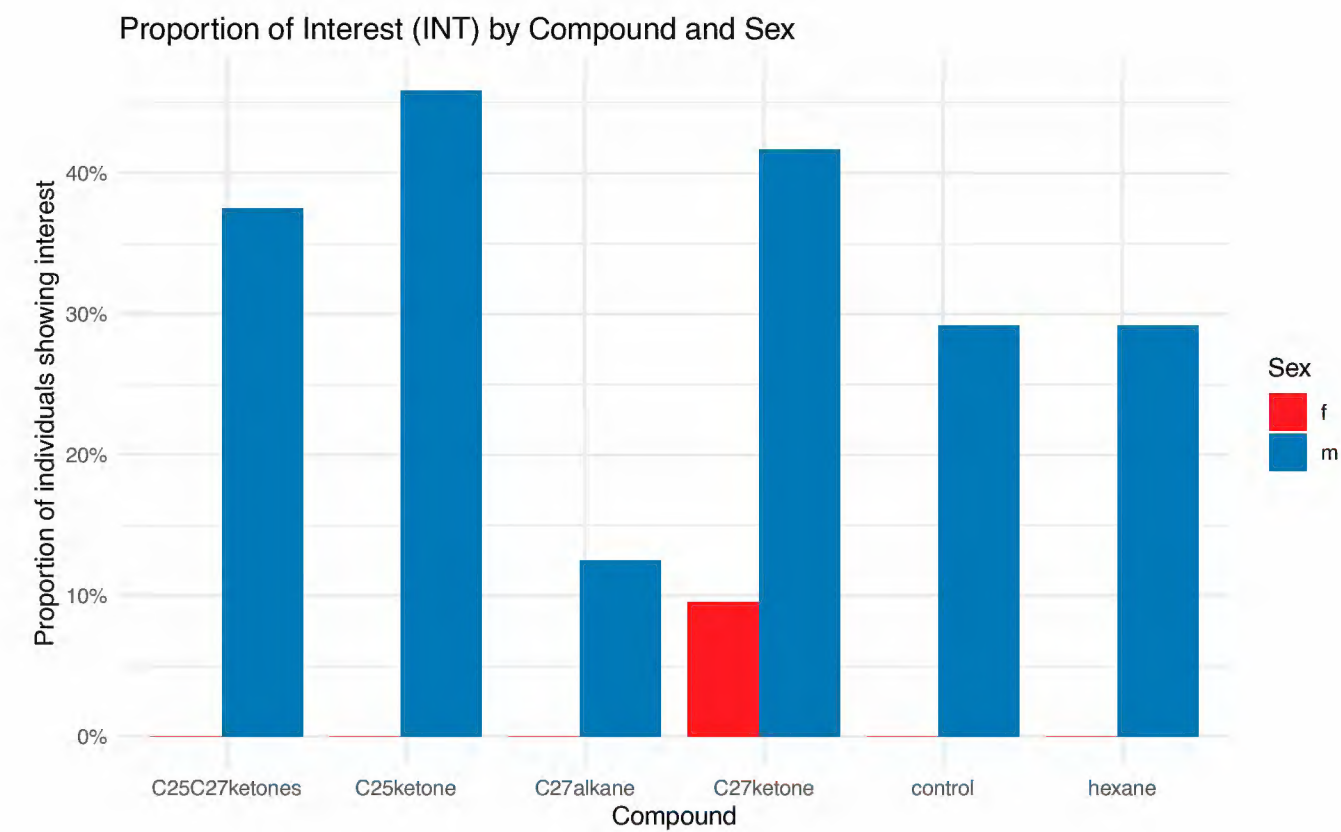


Figure 1. Proportion of male and female individuals showing interest (INT) in the different compounds.

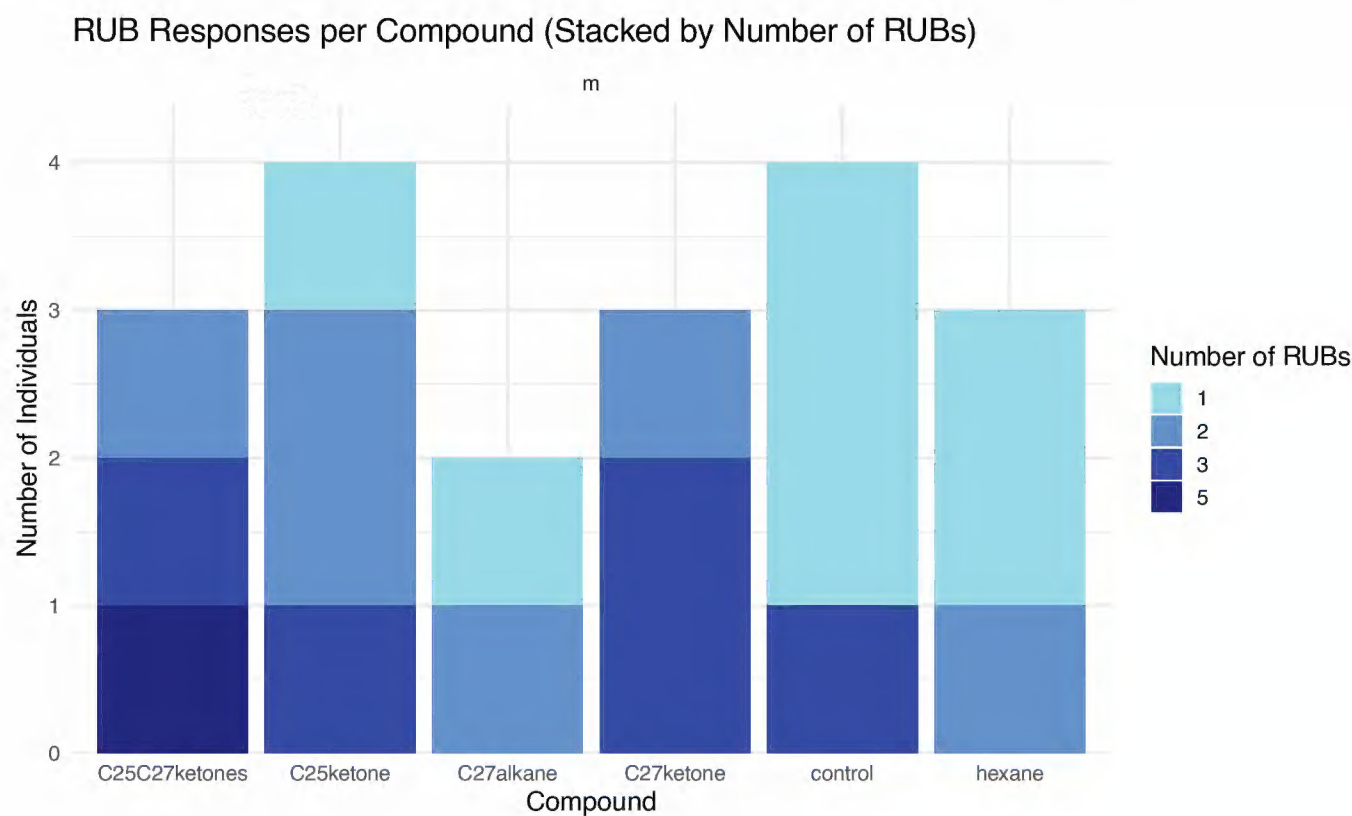


Figure 2. Number of male individuals showing chin-rubbing behavior, with the respective number of chin rubs. No females presented such behavior.

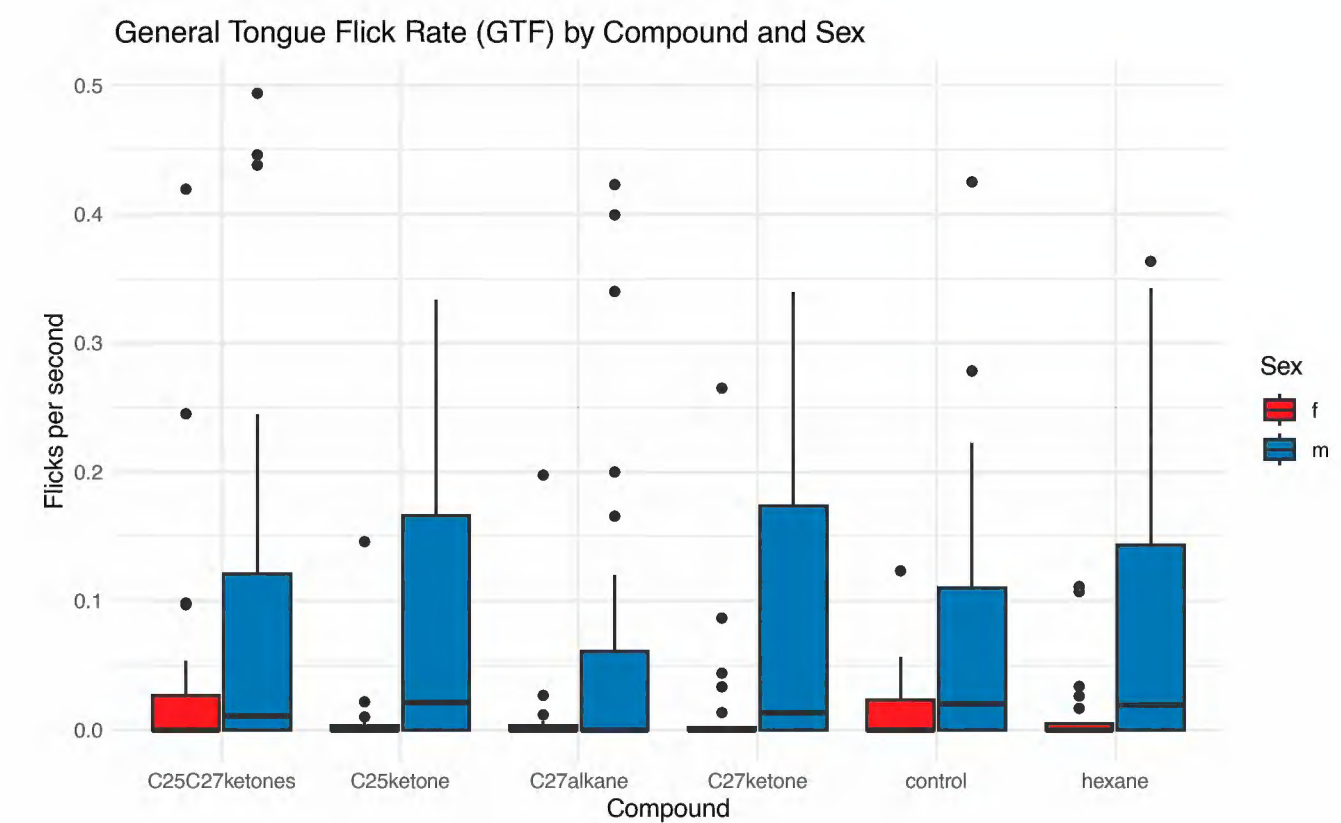


Figure 3. A box plot for general tongue-flick rates (GTF) with minimum, maximum, and average values for male and female individuals.

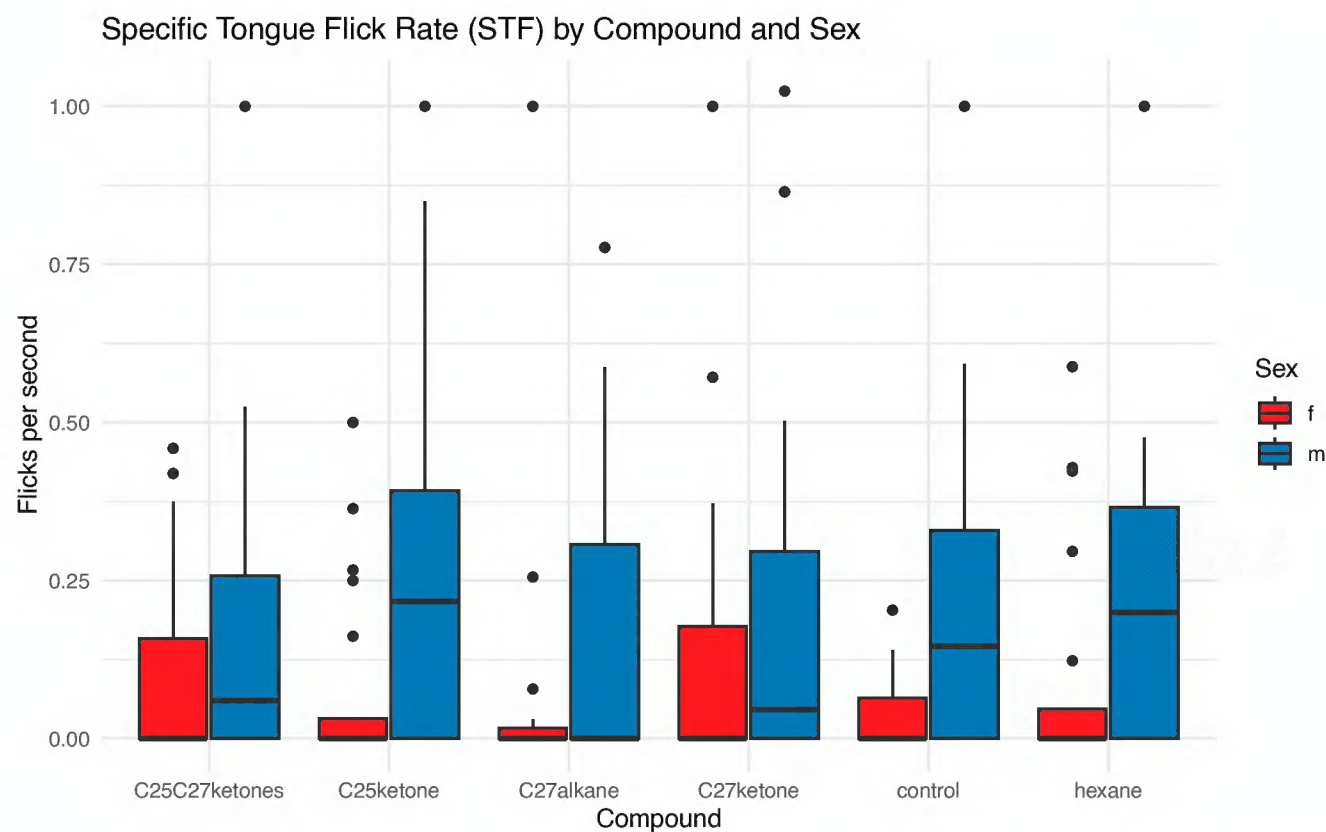


Figure 4. A box plot for specific tongue-flick rates (STF) with minimum, maximum, and average values for male and female individuals.

The multivariate GLMM for males showed statistically significant differences (based on the Highest Posterior Density interval, HPD) for INT between the reactions toward C25 ketone and C27 alkane and between C27 alkane and C27 ketone. A nearly significant difference was found between C25–C27 ketones and C27 alkane. C25 ketone and C27 ketone elicited stronger responses than C27 alkane, with C25–C27 ketones likely provoking similarly strong responses. For RUB, statistically significant differences were observed between reactions to C25–C27 ketones and C27 alkane, between C25–C27 ketones and hexane, and between C25–C27 ketones and the control group. Thus, C25–C27 ketones provoked stronger reactions in males than C27 alkane, hexane, or the control. No statistically significant differences were found among males for GTF and STF (Suppl. material 4).

When compounds were grouped, males showed statistically significant differences between the ketones and alkanes for INT and RUB, with a stronger reaction toward the ketones. No significant differences were found between any of the compound groups for GTF and STF (Suppl. material 4).

In females, statistically significant differences were found for GTF between C25–C27 ketones and C25 ketone, with higher values for C25–C27 ketones. For STF, significant differences were found between C27 alkane and C27 ketone and between C27 ketone and the control group. In both cases, C27 ketone provoked a stronger response in females than C27 alkane and the control. A near-significant difference in STF was observed between C25–C27 ketones and C27 ketone, with elevated tongue-flick rates for C27 ketone. No statistically significant differences were found in females for INT or RUB in response to any of the compounds (Suppl. material 4). However, due to the large number of zeros, some parts of the model did not fully converge (R-hat values > 1.05), so results should be interpreted with caution.

When compounds were grouped, females showed a statistically significant difference for STF between ketones and the control group, with higher values for the ketones.

No significant differences were found in INT, RUB, or GTF among compound groups in females (Suppl. material 4). All statistical results are presented in Suppl. material 2.

Discussion

In the current study, we suggest that two of the ketones found in extracts of *V. ammodytes* by Andonov et al. (2020, 2023), 2-pentacosanone (C25 ketone) and 2-heptacosanone (C27 ketone), indeed provoke sexual attraction to the stimuli in males. They are most likely involved in intraspecific communication and potentially in the composition of the sex pheromone, similarly to species of the genus *Thamnophis* (Mason et al. 1989, 1990). The results also show that the combination of the ketones is more attractive than each of the two ketones alone. Such a trend is also suggested by Mason et al. (1990) for *Thamnophis* spp. Still, the response towards the ketones was not very pronounced, so we suggest that there are other compounds participating in intraspecific sexual behavior.

To investigate whether C25 ketone and C27 ketone have a role in intraspecific communication and are a part of the female sex pheromone composition, we performed a series of behavioral assays. We noticed that nose-horned vipers tended to remain still or defensive in the presence of humans, which was confirmed by the statistical analyses of the videos—individuals started tongue-flicking on average after 291 seconds. Thus, we propose this methodology to be used when working with sedentary ambush predators such as vipers (Saint Girons 1952, 1980, 1997; Neumeyer 1987; Naulleau et al. 1998), and recommend a minimum 10-minute recording duration to capture key behaviors. The only disadvantage of this methodology is that sometimes the individual might move out of the video frame, so a careful balance between visibility (reliable count of tongue-flicks) and frame range (all areas visible) should be maintained. Therefore, when an individual was out of sight for a given period, we removed this period

from the total length of 10 minutes (for GTF) and the period between the first and the last tongue-flicks (for STF), and we calculated the tongue-flick rate accordingly. We assume this did not affect the study results, since the video camera always covered the area of the paper towel and a radius of ca. 20 cm around it, so if an individual went out of the frame, it was likely not interested in the reagent at that time. Also, we analyzed both GTF and STF, so if there were any abnormal records for one of the rates, analyzing the other rate minimized potential errors. Tongue-flick rate alone is not informative enough and should be analyzed along with other key behavioral acts (Burghardt 1967, 1969; Parker and Mason 2011). Thus, we also recorded the presence or absence of interest in the compound dripped on the paper towel (INT) and chin-rubbing behavior (RUB). In addition, Mason et al. (1990) concluded that while males adjust their total number of tongue-flicks and courtship response duration, their tongue-flick rate remains unchanged despite variations in material concentration. Thus, chin-rubbing might be a more reliable sign of sexual attraction than GTF or STF.

The main analyses show a statistically significant difference between males and females. The exploratory behavior was more typical for males, while females tended to stay still for most of the time during the experiments. This was confirmed by statistically significant differences in their reactions to the variables INT and RUB. Thus, for the purposes of the study and for conducting more accurate analyses, we performed separate statistical tests for males and females. This difference suggests confirmation of the assumption that females produce pheromones, attracting males to courtship, which is the case in many species of snakes (Mason et al. 1989, 1990; Greene and Mason 1998, etc.). In addition, the individual effect was shown to be statistically significant. The significant random effect of individual vipers indicates considerable variability in their responses to different compounds. This variability underscores the importance of accounting for individual differences when interpreting behaviors such as interest in the compound, chin-rubbing, general tongue-flick rate, and specific tongue-flick rate.

Considering the aforementioned importance of chin-rubbing as a key behavior in sexual attraction, we suggest that male nose-horned vipers were more attracted to the combination of the two ketones than to the alkanes or the control, based on the results for RUB, and to some extent INT, although GTF and STF did not provide a statistically significant difference. However, since the results did not provide an extremely strong signal for the attraction of males towards the ketones separately, and the GTF and STF rates did not differ substantially, we hypothesize that additional compounds, included in the female sex pheromone, could make females even more attractive. Mason et al. (1989, 1990) describe that long-chain unsaturated ketones are significantly more attractive than saturated ketones for male garter snakes (*T. sirtalis*), even in very small concentrations. However, such compounds were not detected in the skin secretions of *V. ammodytes* by Andonov et al. (2020, 2023).

We also reject the hypothesis that the ketones participate in trailing behavior only, considering that they provoked not only interest in males but also chin-rubbing, which is a clear sign of sexual attraction. The importance of this behavior for vipers is described by Andrén (1982, 1986) for the congeneric *V. berus*. With a detailed description of the main mating behavior phases of the common European viper, *V. berus*—precourtship, courtship, and postmating (Gillingham 1987)—we know that the female produces a male-attractive pheromone leading to male-to-male combats and thereafter, courtship behavior exhibited by the combat winner (Andrén 1986; Andrén and Nilson 1983). Similar behavior with male-to-male combat is recorded for *V. ammodytes* as well (Shine 1978).

Thus, we suggest that either other compounds have a key role in the female attractiveness pheromone, or tests with a combination of a higher number of ketones could provoke a better response. We chose C25 ketone and C27 ketone for the current tests because these are the two most frequent ketones found in female nose-horned vipers, while other ketones, such as 2-nonadecanone, 2-heneicosanone, or 2-tricosanone, were only found in some individuals (Andonov et al. 2020, 2023). As a consequence, even a combination of C25 ketone and C27 ketone only should provoke positive male sexual behavior. However, there are a number of recent publications providing evidence for the other hypothesis, i.e., that there might be another compound in addition to ketones, with a different nature, that is a constituent of the female sex pheromone. Some studies suggest that airborne compounds play a significant role in sexual chemical communication (Aldridge et al. 2005; Shine and Mason 2011; Jellen et al. 2022). Taking this into consideration, we suggest that it is very likely that there are volatile compounds, in addition to the described ketones, that participate in intra-specific sexual communication. However, since behavioral signals, and in this particular case, mating signals, are complex, and various factors (such as visual stimuli) can affect behavioral responses (Shine et al. 2005; Saviola et al. 2012), the weaker response of males to the stimuli might be due to lack of visual stimulus, i.e., presence of an actual female viper. Similarly, the weak response of females might also be due to the same reason, as females would normally respond to initial courtship behaviors in some manner, and these responses might be necessary to trigger the next steps of courting or continued interest by the male. Thus, further research on complex non-chemical stimuli is needed.

We also considered the results for females; however, these findings should be interpreted with caution due to high R-hat values and a large proportion of zero responses. The results indicate that female vipers exhibited higher GTF and STF towards certain ketones in comparison with other compounds, but the results are not entirely consistent. Even though they are rather challenging to interpret, they still suggest some attraction to the ketones. However, there were no significant differences in interest (INT) or chin-rubbing (RUB) behaviors, implying that the attraction is not related to mating behavior. This might mean that females simply recognize the potential presence of another individual if ketones are detected, but without exhibiting mating behaviors.

Assuming ketones are key components of female pheromones, their presence in male extracts is puzzling (Andonov et al. 2020, 2023). Similar chemical secretions in both males and females are observed also in *Vipera berus* (Linnaeus, 1758) (Van Moorleghe et al. 2020; Andonov et al. 2023). One explanation of such observations can be an argument with the presence of “she-males” in *V. ammodytes* similar to those described for *T. sirtalis* (Mason and Crews 1985, 1986). However, this explanation is dubious considering the different mating behaviors between these two species—*T. sirtalis* is characterized by specific mating ball behavior where tens or even hundreds of males gather over and try to copulate with one female (Mason and Crews 1985, 1986), while *V. ammodytes* is known for its male-to-male combats (Shine 1978), although recent findings show the presence of group mating in the species with potential participation of “she-males” as well (Čubrić and Crnobrnja-Isailović 2023). Therefore, there is an incentive for male *T. sirtalis* to produce female pheromones and thus, “trick” the other males who get exhausted while trying to copulate with a certain “she-male,” leading to a mating advantage for this “she-male” (Shine et al. 2000, 2001). Perhaps male nose-horned vipers produce female pheromones to attract other males and provoke them to fight one another so they get tired, which will give the “she-male” a possible advantage in potential male-to-male combat with the previous winner or even avoid male-to-male combat at all. Thus, similar to the “she-males” in *T. sirtalis*, male vipers who produce female pheromones could have higher chances for successful mating. Another explanation might be related to the known scent deposition in *Malpolon monspessulanus* (Hermann, 1804) (de Haan 1982). It is possible that males mark different places with scents similar to female pheromones in order to distract other males and confuse them in their search for females. The marking hypothesis appears plausible, considering that ketones are heavy and non-volatile, so they can be easily deposited to different substrates.

Another outstanding question is why some females refrain from producing the ketones presumed to function in mate attraction (Andonov et al. 2020, 2023). The reproductive behavior patterns of European vipers may explain this. Female European vipers usually do not reproduce each year but once every 1–2 or even 3 years (Saint Girons and Kramer 1963; Beshkov 1977; Nilson 1981; Naulleau and Bonnet 1995; Bonnet and Naulleau 1996; Luiselli and Zuffi 2002). This is because viviparity is a very energy-dependent process for female vipers, and they need a high number of reserves for successful reproduction (Bonnet et al. 1994; Bonnet and Naulleau 1995; Naulleau and Bonnet 1996; Baron et al. 2012; Bauwens and Claus 2019). Females without enough reserves either do not initiate reproduction or have very low reproductive success (Bonnet et al. 1994; Dyugmedzhiev et al. 2018). It is possible that female *V. ammodytes* with insufficient reserves for successful reproduction do not produce ketones, which makes them unattractive for males. A similar pattern has been documented for *T. sirtalis*, where the ketone profiles differ between larger and smaller females, with the first being much more attractive to males than the latter (LeMaster

and Mason 2002; Shine et al. 2003). However, this hypothesis should be further tested, and a body condition correlation with ketone production in vipers should be analyzed.

The different body conditions of male individuals might affect their behaviors and responses to different stimuli. As the current sample size would not allow for reliable conclusions on the effect of body size, we did not test for such a correlation, but further studies on the matter are required. In addition, the variation of hormonal levels might also alter snakes’ activity, so a parallel study on blood samples of the individuals during or after behavioral experiments can be a useful addition for future studies.

Conclusion

In the present study, we demonstrate that male *Vipera ammodytes* are the active participants in intersexual communication, whereas females exhibit comparatively passive behavior. Our findings suggest that 2-pentacosanone and 2-heptacosanone, identified in *V. ammodytes* extracts, elicit courtship-related behaviors in males and likely play a role in intraspecific communication, potentially contributing to the sex pheromone. Additionally, the combination of these ketones proved more attractive than either compound alone. However, the response was not strongly pronounced, indicating that other compounds may also influence intraspecific sexual behavior. We also speculate that long-chained ketones might be used by males for intrasexual communication, potentially providing a reproductive advantage, although this should be further investigated.

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Supplementary material 1

Descriptive statistics of the four key variables analyzed—interest (INT), chin rubbing (RUB), general tongue-flick rates (GTF), and specific tongue-flick rates (STF)

Authors: Kostadin Andonov, Angel Dyugmedzhiev, Simeon Lukanov, Emiliya Vacheva, Georgi Popgeorgiev, Daniela Nedeltcheva-Antonova, Borislav Naumov

Data type: xlsx

Explanation note: The dataset presents the descriptive statistics of the four key variables analyzed—interest (INT), chin rubbing (RUB), general tongue-flick rates (GTF), and specific tongue-flick rates (STF)—including minimum, maximum, and average values for both male and female individuals.

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Link: <https://doi.org/10.3897/herpetozoa.38.e151358.suppl1>

Supplementary material 2

Results of the statistical analyses performed

Authors: Kostadin Andonov, Angel Dyugmedzhiev, Simeon Lukanov, Emiliya Vacheva, Georgi Popgeorgiev, Daniela Nedeltcheva-Antonova, Borislav Naumov

Data type: docx

Explanation note: The supplementary material presents the results of all of the performed GLMMs with the respective confidence intervals (CI).

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Link: <https://doi.org/10.3897/herpetozoa.38.e151358.suppl2>

Supplementary material 3

Behavioral data used for the statistical analyses

Authors: Kostadin Andonov, Angel Dyugmedzhiev, Simeon Lukanov, Emiliya Vacheva, Georgi Popgeorgiev, Daniela Nedeltcheva-Antonova, Borislav Naumov

Data type: csv

Explanation note: The dataset includes the recorded interest (INT), chin rubbing (RUB), general tongue-flick rates (GTF), and specific tongue-flick rates (STF) for each behavioral test.

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Supplementary material 4

Graphs of the statistical results

Authors: Kostadin Andonov, Angel Dyugmedzhiev, Simeon Lukanov, Emiliya Vacheva, Georgi Popgeorgiev, Daniela Nedeltcheva-Antonova, Borislav Naumov

Data type: pdf

Explanation note: The graphs represent the confidence intervals (CI) resulting from the performed statistical analyses, with the results of statistical significance marked in red.

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